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Citation for published version:

Staderini, M, Gambardella, A, Lilienkamp, A & Bradley, M 2018, 'A Tetrazine-Labile Vinyl Ether Benzyloxycarbonyl Protecting Group (VeZ): An Orthogonal Tool for Solid-Phase Peptide Chemistry', *Organic letters*, vol. 20, no. 11, pp. 3170-3173. <https://doi.org/10.1021/acs.orglett.8b00898>

Digital Object Identifier (DOI):

[10.1021/acs.orglett.8b00898](https://doi.org/10.1021/acs.orglett.8b00898)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Organic letters

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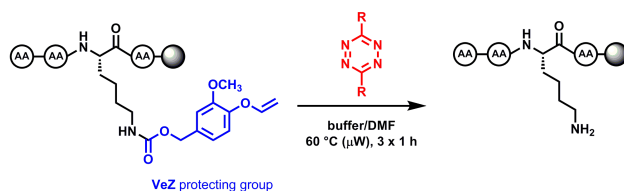
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A Tetrazine-Labile Vinyl Ether Benzyloxycarbonyl Protecting Group (VeZ) – An Orthogonal Tool for Solid-Phase Peptide Chemistry

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ABSTRACT: The vinyl ether benzyloxycarbonyl (VeZ) protecting group is selectively cleaved by treatment with tetrazines *via* an inverse electron-demand Diels–Alder reaction. This represents a new orthogonal protecting group for solid-phase peptide synthesis, with Fmoc-Lys(VeZ)-OH as a versatile alternative to Fmoc-Lys(Alloc)-OH and Fmoc-Lys(Dde)-OH for the synthesis of cyclic peptides, as demonstrated by the synthesis of melanotan II (MTII) and a constrained BAD BH3 peptide analogue.

Peptides are valuable scaffolds due to their intrinsic biological and chemical properties.¹ Cyclic peptides often show good *in vivo* stability, while demonstrating enhanced biological activities compared to their linear counterparts.² Given the growing interest in these constrained molecules as therapeutic and biomedical tools, there is a demand for efficient synthetic approaches.³ In solid-phase peptide synthesis (SPPS), protecting groups orthogonal to Fmoc are typically used to temporarily mask side-chain functionalities to enable controlled synthesis *via* the so-called Fmoc/^tBu approach.^{4–6} For the generation of cyclic or complex peptides, such as those decorated on their side-chains,⁷ the demands are more complex with the need for another tier of orthogonality, typically provided by Alloc⁸ or Dde (or *iv*Dde),⁹ both which have their limitations. The cleavage of the Alloc group requires the use of palladium salts, potentially complicating the purification of peptides aimed at biological and medical applications, while the standard conditions used for the removal of the Dde (2–5% hydrazine in DMF) are not compatible with the Fmoc group and will reduce the Alloc group, furthermore, the Dde moiety can undergo migration.^{10, 11} Therefore, efforts have been made to develop a variety of other orthogonal cleavage strategies that offer mild cleavage conditions for the selective deprotection of side-chain groups.^{12–17}

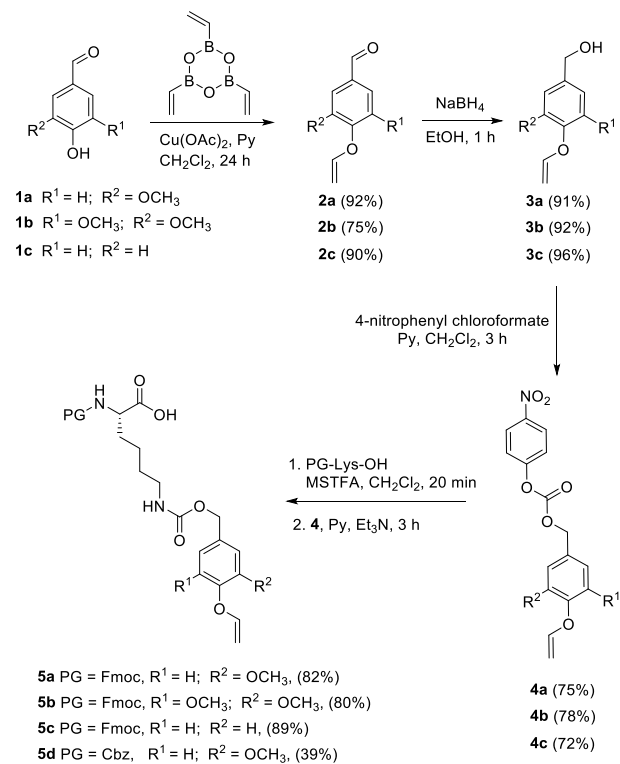
Inverse electron-demand Diels–Alder (invDA) reactions between tetrazines and electron-rich dienophiles have been widely used in bioconjugation chemistry.^{18, 19} Recently, they have found applications in drug delivery/prodrug activation^{20–23} and protein activation chemistries,²⁴ with the tetrazine acting as a trigger, which upon the invDA reaction starts a cascade process resulting in the liberation of the cargo and formation of pyridazine as a side product. For example, Robillard developed a biorthogonal system using a *trans*-cyclooctene as a

strained dienophile that allowed drug release through an elimination reaction mediated by a tetrazine.²³ Li used an invDA-mediated deprotection strategy for bioorthogonal protein activation where the ϵ -amino group of a catalytic lysine residue was likewise modified with a *trans*-cyclooctene making the target protein inactive until uncaged by a tetrazine.²⁴ Our group reported a polymeric drug delivery system, which featured a vinyl ether as a dienophile that, upon activation, uncaged a phenol(ate) that underwent subsequent 1,6-elimination and cargo liberation.²⁰ Herein, we introduce the vinyl ether benzyloxy carbonyl (VeZ) group as a tetrazine-cleavable protecting group that was used to mask the ϵ -amino group on lysine, with Fmoc-Lys(VeZ)-OH used as a Fmoc/^tBu-orthogonal protected building block in the solid-phase synthesis of cyclic peptides.

Three VeZ derivatives were synthesized with zero, one or two methoxy groups incorporated into the phenyl ring as increasing electron density was envisioned to promote the invDA reaction by reducing the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) gap with the diene.^{19, 25} The VeZ moiety was prepared *via* vinylation of the phenolic hydroxy group in the corresponding aldehydes **1a–c** using the vinyl boronic anhydride pyridine complex and copper(II) acetate,²⁶ followed by reduction of the aldehydes **2a–c** with NaBH₄ to give the benzylic alcohols **3a–c**, which were subsequently transformed into the corresponding carbonates **4a–c** by treatment with 4-nitrophenyl chloroformate (Scheme 1). Carbonates **4a–c** were coupled onto Fmoc-Lys-OH and Cbz-Lys-OH, with transient protection of the carboxyl group with MSTFA,²⁷ allowing incorporation of the VeZ protecting group on the side chain through a carbonate linkage to give **5a–d** (24–55% over four steps) (Scheme 1).

Treatment of the VeZ protecting group with a tetrazine was envisioned to trigger a cascade process starting with the removal of the vinyl ether group to liberate the phenol(ate), followed by amino liberation through a 1,6-elimination reaction (Figure 1). Initially, **5a** (5.6 mM) was treated with the widely used tetrazine, 3,6-di-2-pyridyl-1,2,4,5-tetrazine²⁰ (17 mM), in DMF at various temperatures (up to 60 °C); however, no reaction of the vinyl ether was observed after 24 h (Table 1).

Scheme 1. Synthesis of the VeZ protected lysines **5a–d**



Since water is known to dramatically accelerate Diels–Alder reactions, perhaps by promoting hydrophobic interactions between the dienophile and tetrazine or acting as a general acid,¹⁹ **5a** was treated with tetrazine in 10% H₂O/DMF (v/v) at 60 °C. Under these conditions, 80% conversion of the vinyl compound into the phenol derivative **6** was observed (as determined by HPLC-MS), with **6** isolated in 65% yield after column chromatography. The introduction of two methoxy groups (compound **5b**) resulted in slower conversion into the corresponding phenolate, whereas **5a** (one methoxy group) and **5c** (no methoxy groups) showed comparable reactivity in reactions monitored by ¹H NMR (Supporting Information (SI), Figure S1–S3).

It was somewhat surprising that the phenol intermediate **6** was isolable. The subsequent 1,6-elimination from **6** was promoted by increasing the water content in the reaction mixture; however, even 50% H₂O/DMF (v/v) only resulted in a 1:1.4 ratio of the phenol **6** and the desired Fmoc-Lys-OH (Table 1). Protonation of the tetrazine and its consequent increased electron deficiency is known to enhance reaction rates in invDA reaction chemistry,¹⁹ while a lower pH would also promote

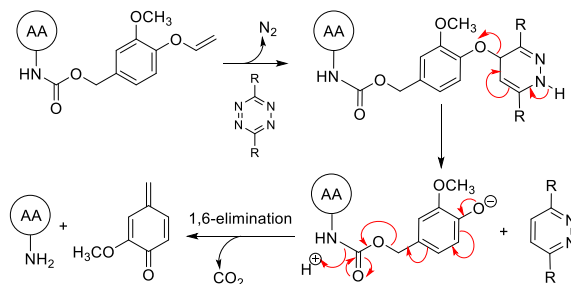


Figure 1. Mechanism of tetrazine-mediated vinyl ether decaging and amine liberation.

Table 1. Tetrazine-mediated deprotection of Fmoc-Lys(VeZ)-OH **5a**^a

entry	temp (°C)	solvent	time (h)	conv (%)	ratio 6 :Lys ^d
1	rt	DMF	24	0	—
2	40	DMF	24	0	—
3	60	DMF	24	0	—
4	rt	10% H ₂ O/DMF	18	2	1:0
5	40	10% H ₂ O/DMF	18	58	1:0
6	60	10% H ₂ O/DMF	18	80	1:0
7	60	25% H ₂ O/DMF	18	92	1:1
8	60	50% H ₂ O/DMF	18	100	1:1.4
9	60	50% buffer ^b /DMF	18	100	0:1
10	40 ^c	50% buffer/DMF	2	33	2:1
11	40 ^c	50% buffer/DMF	4	43	2:1
12	60 ^c	50% buffer/DMF	1	48	1:1
13	60 ^c	50% buffer/DMF	2	58	1:3
14	60 ^c	50% buffer/DMF	4	71	1:10

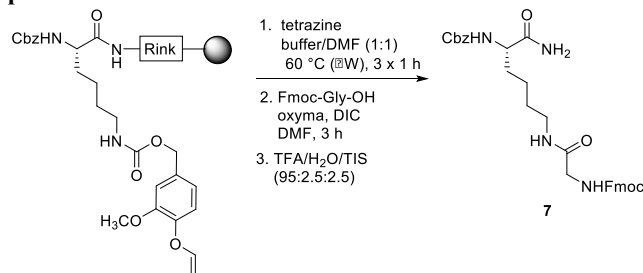
^aReaction conditions: Fmoc-Lys(VeZ)-OH **5a** (5.6 mmol), 3,6-di-2-pyridyl-1,2,4,5-tetrazine (17 mmol). ^bSodium citrate buffer (100 mM, pH 5). ^cThe reaction was carried out under μ W irradiation. ^dFmoc-Lys-OH.

protonation of the carbamate, thereby enhancing the 1,6-elimination reaction. Thus, when the reaction with the tetrazine was carried out at 60 °C for 18 h using a 1:1 (v/v) mixture of pH 5 sodium citrate buffer and DMF, full conversion of compound **5a** into the corresponding ϵ -amino free lysine was observed (Table 1, entry 9). This reaction could be accelerated by performing the deprotection reaction under microwave (μ W) irradiation conditions (60 °C for 4 h) giving the VeZ

deprotected lysine as the main product (longer irradiation times did not improve conversion due to rapid degradation of the tetrazine).

These optimized VeZ deprotection conditions were directly transferred onto the solid support using the water compatible resin ChemMatrix (loading 1 mmol/g, 35–100 mesh, loaded with an Fmoc-Rink-amide linker) that was subsequently functionalized with Cbz-Lys(VeZ)-OH (**5d**) (Scheme 2). Using 3 × 1 h deprotection cycles with fresh tetrazine solution. Subsequent coupling with Fmoc-Gly-OH and cleavage of the resin, gave the dipeptide **7** in quantitative yield, showing the efficient cleavage of the vinyl ether and the subsequent 1,6-elimination reaction.

Scheme 2. Tetrazine-mediated deprotection on the solid-phase



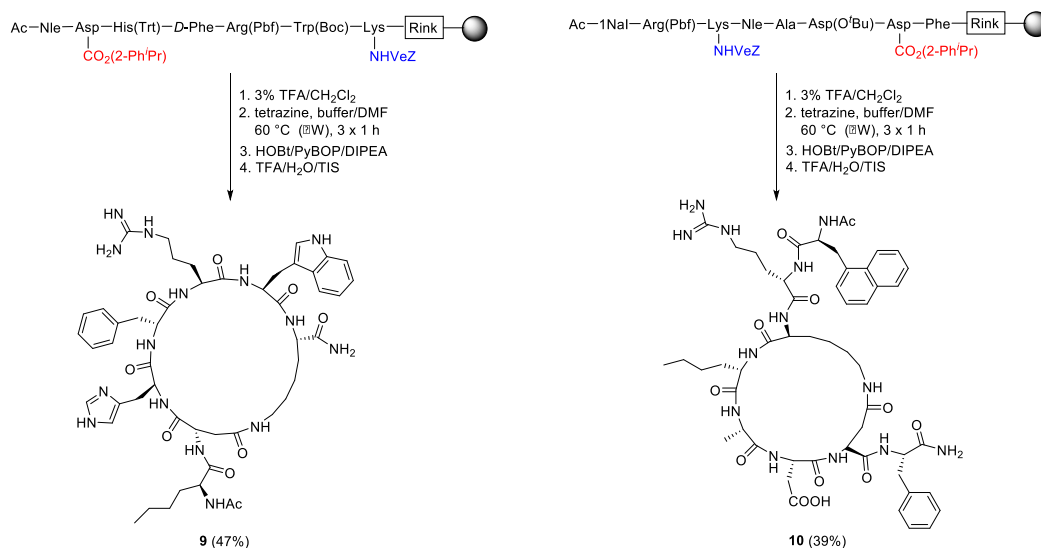
To explore possible epimerization under these deprotection conditions, two diastereoisomeric peptides Ac-Phe-Lys(Ac)-NH₂ **8a** and Ac-phe-Lys(Ac)-NH₂ **8b** were synthesized on a ChemMatrix resin that had been functionalized with a Rink linker, with subsequent coupling of Fmoc-Lys(VeZ)-OH. Upon deprotection of the Fmoc group, either Fmoc-Phe-OH or Fmoc-phe-OH were coupled to the lysine followed by the cleavage of the VeZ group using the tetrazine-mediated proto-

col described above. Acetylation of the free amino groups and cleavage from the resin gave the diastereoisomers **8a** and **8b** that were clearly resolved by HPLC and HPLC analysis showed no evidence of epimerization (Figure S5).

In order to evaluate the orthogonality and compatibility between the VeZ group and other protecting groups commonly used in peptide chemistry (e.g. Fmoc, Dde, Allyl, Alloc, Boc), as well as standard cleavage protocols, stability studies were carried out in solution alongside **5a** and **5d**. The VeZ group was readily cleaved from the lysine side chain under strongly acidic conditions (95% TFA), but was stable in 2% TFA and 20% HFIP for more than 3 h (Table S1). The VeZ group was also compatible with standard Fmoc-based SPPS conditions (carbodiimide-activated couplings and 20% piperidine in DMF), Alloc removal (Pd(PPh₃)₄, PhSiH₃ in DMF), Dde removal (2% hydrazine in DMF and NH₂OH·HCl/imidazole in NMP/DMF), confirming its suitability in peptide synthesis. However, the Alloc group was not stable upon treatment with tetrazine (4 h μW irradiation at 60 °C), whereas the *S*-trityl group was observed to be robust under these conditions (Table S2).

To test its versatility, Fmoc-Lys(VeZ)-OH **5a** was applied to the synthesis of two cyclic peptides, melanotan II (**9**) (MTII)²⁸ and a constrained BAD BH3 peptide analogue **10**,²⁹ both examples of side-chain to side-chain lactam-bridge peptides (Scheme 3). MTII **9** (Ac-Nle-c[Asp-His-phe-Arg-Trp-Lys]-NH₂) is a potent agonist of melanocortin receptors,²⁸ while **10** (Ac-1Nal-Arg-c[Lys-Nle-Ala-Asp-Asp]-Phe-NH₂) is a potential anticancer agent that exerts its activity by inhibiting Bcl-2.²⁹ The fully protected linear peptides were synthesized on a Rink-amide functionalized ChemMatrix resin using a Fmoc-based solid-phase chemistry with DIC and Oxyma as the coupling combination and Fmoc-Lys(VeZ)-OH **5a** as a building block. After the selective cleavage of the 2-phenylisopropyl group (*O*-2-Ph^tPr) from the side chain of aspartic acid using 3% TFA in CH₂Cl₂, the VeZ group was cleaved using the

Scheme 3. Side chain to side chain cyclization of linear peptides using the tetrazine-cleavable VeZ as an orthogonal side-chain protecting group



tetrazine-mediated protocol (3 × 1 h of dipyrityl tetrazine (30 mM) in 1:1 (v/v) sodium citrate buffer and DMF at 60 °C, μ W). On-resin cyclization was achieved using Py-BOP/HOBt/DIPEA in DMF and was followed by deprotection of the side chains and cleavage from the solid support by TFA, followed by purification by preparative HPLC, giving the cyclic peptides **9** and **10** in 47% and 39% isolated yields, respectively.

In conclusion, the benzyloxycarbonyl vinyl ether (VeZ) protecting group has been introduced as a temporary masking group for amino functionalities during solid-phase peptide synthesis. It can be removed by treatment with a tetrazine via devinylation and subsequent 1,6-elimination chemistries. The VeZ protecting group was stable under standard Fmoc-based SPPS conditions and could be selectively removed under relative mild conditions without affecting other common side chain protecting groups or the Rink-amide linker. The applicability of the approach was demonstrated by the solid-phase synthesis of two biologically relevant cyclic peptides. It is interesting to note that the tetrazine-mediated inverse Diels-Alder reaction was hugely accelerated by the presence of even 10% water and that the 1,6-elimination reaction of the “safety-catch linker”³⁰ was not spontaneous and was promoted by acidic conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting Figures/Tables, experimental procedures, compound characterization, and NMR spectra (PDF).

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Author Contributions

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by the European Research Council (Advanced Grant ADREEM ERC-2013-340469) and the EPSRC funded Implantable Microsystems for Personalised Anti-Cancer Therapy (IMPACT) Programme (Grant re. EP/K034510/1).

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